Antibodies against type II phospholipase A_2 prevent renal injury due to ischemia and reperfusion in rats

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Abstract **This study was performed to determine the involvement of type II phospholipase A_2 (PLA_2-II) in renal injury caused by ischemia and reperfusion. Ischemia and reperfusion significantly elevated levels of blood urea nitrogen and serum creatinine in rats. These increases were significantly reduced by i.v. administration of rabbit IgG F(ab′)2 fragments against rat PLA_2-II. Increased levels of acid-stable PLA_2 activity in the kidney were caused by ischemia and reperfusion, and were suppressed by administration of anti-PLA_2-II F(ab′)2. Increased levels of myeloperoxidase activity, a marker of neutrophil infiltration, in the kidney were also reduced after anti-PLA_2-II F(ab′)2 treatment. These results suggest that PLA_2-II plays a pivotal role in pathogenesis of ischemia and reperfusion injury through induction of neutrophil infiltration.**

Key words: Phospholipase A_2; Ischemia; Myeloperoxidase; Renal failure

1. Introduction

Phospholipase A_2 (PLA_2), which hydrolyzes the *sn*-2 ester bonds of phospholipids to yield free fatty acids and lysophospholipids, is not only involved in lipid metabolism but is critical to signal transduction and production of proinflammatory lipid mediators [1]. Three types of Ca^{2+}-dependent PLA_2, types I, II and IV, have been identified in rat kidney tissue [2,3]. Types I and II are 14 kDa proteins, secreted from various cells such as macrophages, endothelial cells, renal mesangial cells, and fibroblasts, and exhibit no specificity for the fatty acid at the *sn*-2 position of phospholipids [1]. Type IV PLA_2 is an 85 kDa protein, ubiquitously expressed in cytosol, which exhibits a preference for arachidonic acid at *sn*-2. Type I PLA_2 is localized in pancreatic juice and its main role is digestion of phospholipids in food. Type II PLA_2 (PLA_2-II) has been detected in a wide variety of inflammatory patients, such as rheumatoid arthritis, septic shock, and pancreatitis, and is therefore implicated in the inflammation process [4].

Neutrophil infiltration during ischemia and reperfusion has been implicated as a key mediator in renal tissue injury through release of oxygen radicals, proteases, arachidonic acid metabolites and other cytotoxic species [5]. On the other hand, Ca^{2+}-dependent, low molecular PLA_2 activity increases in experimental models of kidney ischemia [6,7]. In connection with those findings, PLA_2-II exogenous added to cell culture media activates CD11b/CD18 expression, degranulations, superoxide production and generation of arachidonic acid metabolites in neutrophils [2,8–11]. From those observations, it is surmised that PLA_2-II plays a critical role in renal injury due to ischemia and reperfusion through neutrophil activation.

In the present study, the involvement of PLA_2-II in acute renal failure was evaluated by the effects of antibodies against PLA_2-II on renal injury due to ischemia and reperfusion in rats.

2. Materials and methods

2.1. Preparation of F(ab′)2 fragments of rabbit IgG against rat PLA_2-II

Platelets from about 100 Wistar rats (body weight 400–500 g) were stimulated with thrombin, and PLA_2-II was purified as described by Horigome et al. [12]. The purified protein gave a single protein band on SDS-PAGE, the molecular mass of which was estimated to be 14 kDa, corresponding to rat PLA_2-II.

F(ab′)2 fragments of rabbit IgG against rat PLA_2-II (anti-PLA_2-II F(ab′)2) were prepared as described elsewhere [10]. Briefly, rabbits (KBL:JW) were immunized 4 times with 0.1 mg of purified rat PLA_2-II emulsified in Freund's complete adjuvant. The serum IgG was purified with protein A-Sepharose column (Pharmacia LKB Biotechnology, Piscataway, NJ) and digested with pepsin. The F(ab′)2 fragment was purified by Sephacryl S-200HR gel filtration column (Pharmacia). The F(ab′)2 solution was adjusted to 1 mg/ml in saline. Non-immune F(ab′)2 was prepared from normal rabbit serum by the same way.

2.2. Surgical procedures

Male Wistar rats (body weight about 300 g, Japan SLC, Hamamatsu) were used in the following experiments, which were performed in accordance with the regulations of the Animal Ethical Committee of Yamanouchi Pharmaceutical Co. Ltd. The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and the body temperature was maintained at 37°C with a warming pad. A jugular venous catheter was inserted in all animals for drug administration and blood sampling. Both kidneys were exposed, and the renal arteries and veins were occluded bilaterally for 60 min with non-traumatic vascular clamps. Reperfusion was initiated by removing the clamps, and the abdomen was closed.

Sham-operated control animals (eight rats) without renal ischemia formed group I. The rats with renal ischemia were divided into three groups. Groups II, III, and IV were given saline, non-immune F(ab′)2 and anti-PLA_2-II F(ab′)2, respectively. Non-immune F(ab′)2 or anti-PLA_2-II F(ab′)2 was i.v. administered in a bolus dose of 5 mg/5 ml saline/kg at the time of reperfusion, followed by i.v. infusion at a rate of 5 mg/5 ml saline/kg/h for the subsequent 6 h. This dosage was based on the following experiments. Pharmacokinetic analysis estimated the half-life of F(ab′)2 in rats to be 11 h. This bolus plus infusion dosage yielded a concentration of 200 μg/ml in plasma and provided a concentration of >10 μg/ml during the in vivo experimental period (48 h), whereas 100% inhibition of PLA_2-II was achieved in vitro (Fig. 1).

Venous blood was drawn from the jugular vein 48 h after reperfusion. Levels of blood urea nitrogen (BUN) and serum creatinine of those samples were measured with diagnostic kits (BUN Test Wako

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Abbreviations: PLA_2-II, type II phospholipase A_2; MPO, myeloperoxidase; BUN, blood urea nitrogen

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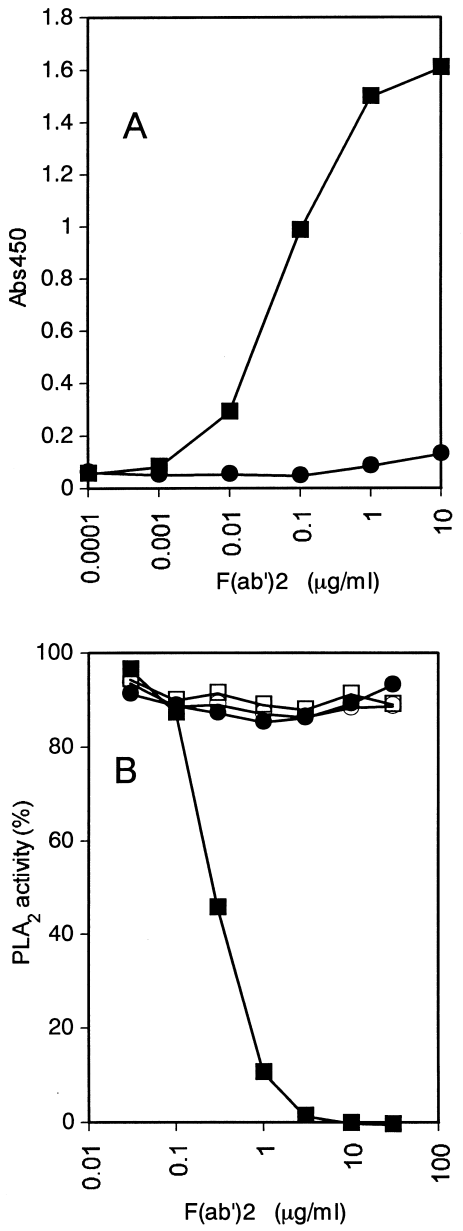

Fig. 1. Specificity of anti-PLA_2-II F(ab′)2. A: Binding capacity was investigated by ELISA assay using plates coated with 5 ng/well of rat PLA_2-II (■) or porcine PLA_2-I (●). B: Anti-PLA_2-II F(ab′)2 (closed symbols) or non-immune F(ab′)2 (open symbols) was incubated with 0.5 ng of rat PLA_2-II (■,□) or porcine PLA_2-I (●,○). Residual PLA_2 activities are expressed as percentages of control not treated with any F(ab′)2.

and Creatinine Test Wako; Wako Pure Chemical Co. Ltd., Osaka, Japan).

Animals were killed 48 h after reperfusion, and both kidneys were excised. The left kidneys were fixed in formalin and stained with hematoxylin and eosin. The right kidneys were stored at −80°C for measurement of PLA_2 and myeloperoxidase activities as described below.

2.3. Assay of PLA_2 and myeloperoxidase (MPO) activities

Kidneys were homogenized in 10 ml of 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA with a Potter-type homogenizer. For measurement of PLA_2 activity, 5 ml of kidney homogenate was extracted with 0.18 N H_2SO_4 on ice for 60 min as described by Märki and Franson [13]. The extracts were centrifuged at 15000 rpm at 4°C for 30 min. The supernatants were dialyzed against 50 mM Tris-HCl (pH 7.4) containing 0.2 M NaCl. PLA_2 activities in the acid extracts were measured using [^{3}H]oleic acid labeled, autoclaved *Escherichia coli* as substrate, as described previously [10].

For measurement of MPO activity, 5 ml of kidney homogenate were extracted in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, and the MPO activities in the extracts were measured using tetramethylbenzidine as substrate as described by Laight et al. [14].

Protein concentrations of kidney extracts were assessed with the Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA).

2.4. Statistical analysis

All data are indicated as the means ± S.E.M. Statistical analysis was performed by Student's *t*-test for comparisons between two groups.

3. Results

3.1. Specificity of rabbit anti-PLA_2-II F(ab′)2

I.v. administration of anti-PLA_2-II IgG, but not its F(ab′)2 fragment, greatly decreased platelets in rats (data not shown), which may be due to effector functions of the Fc moiety of IgG. Therefore, the F(ab′)2 fragment but not the whole IgG molecule was used for in vivo studies.

Specificity and binding capacity of anti-PLA_2-II F(ab′)2 were investigated by ELISA using plates coated with rat PLA_2-II or porcine PLA_2-I (Fig. 1A). Anti-PLA_2-II F(ab′)2 bound to PLA_2-II but not to PLA_2-I. To determine the effect on the PLA_2 activity, anti-PLA_2-II F(ab′)2 or non-immune F(ab′)2 were pre-incubated with rat PLA_2-II or porcine PLA_2-I at room temperature for 60 min. Residual enzyme activities were measured with [^{3}H]oleic acid labeled, autoclaved *E. coli* as substrate (Fig. 1B). Anti-PLA_2-II F(ab′)2 inhibited the enzyme activity of PLA_2-II but not PLA_2-I, while non-immune F(ab′)2 did not inhibit either PLA_2-II or

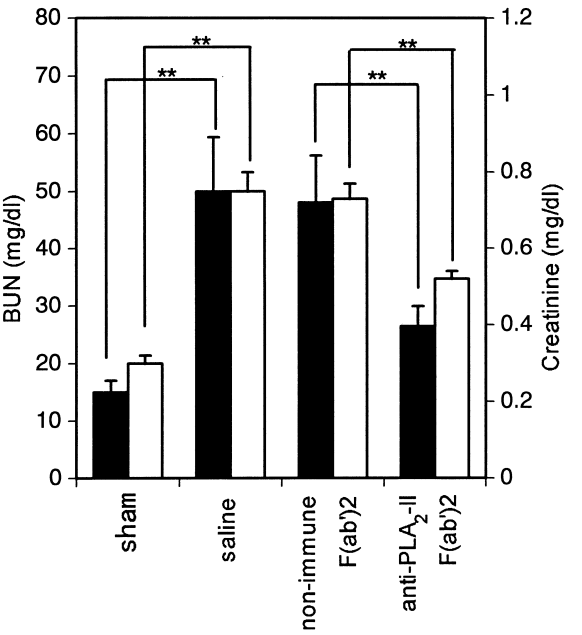

Fig. 2. Effects of anti-PLA_2-II F(ab′)2 on rat renal functions 48 h after ischemia and reperfusion. Levels of BUN and serum creatinine were measured in the blood samples of the sham-operated control animals ($n=8$), saline, non-immune F(ab′)2- and anti-PLA_2-II F(ab′)2-treated rats ($n=10$) with ischemia and reperfusion. Closed bars represent BUN levels and open bars represent serum creatinine levels. Those levels are expressed as means ± S.E.M. $^{**}P<0.01$.

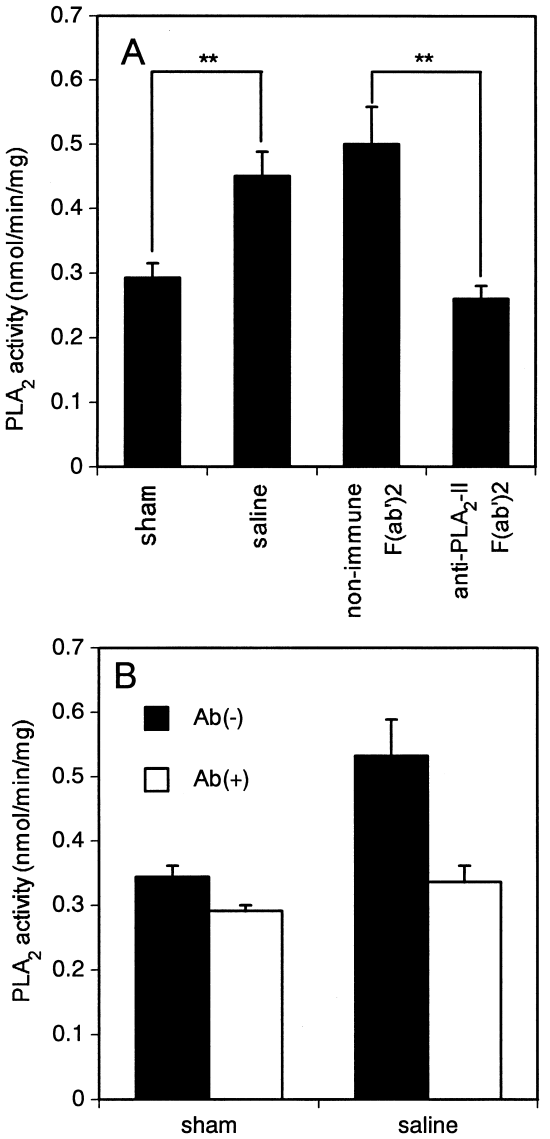

Fig. 3. PLA_2 activity in the kidneys 48 h after ischemia and reperfusion. A: PLA_2 activity of the kidney acid extracts are expressed as means ± S.E.M. ($n = 10$). $^{**}P < 0.01$. B: PLA_2 activity of the kidney extracts of sham-operated control rats and saline-treated rats with ischemia and reperfusion, which were pre-incubated without (closed bars) or with (open bars) 100 μg/ml anti-PLA_2-II F(ab′)2 ($n = 5$).

PLA_2-I. Thus, the rabbit anti-PLA_2-II F(ab′)2 used here blocks the enzyme activity of PLA_2-II with high specificity.

3.2. Effect of anti-PLA_2-II F(ab′)2 on rat renal injury due to ischemia and reperfusion

The renal arteries and veins of rats were occluded bilaterally with clamps for 60 min and the kidneys were then reperfused by removing the clamps. The levels of BUN and serum creatinine were assessed 48 h after ischemia and reperfusion (Fig. 2). The BUN values in saline-treated animals with ischemia and reperfusion (group II) was 50.0 ± 9.4 mg/dl vs. 15.0 ± 2.0 mg/dl ($P < 0.01$) in sham-operated animals (group I). The creatinine values in group II was 0.75 ± 0.05 mg/dl vs. 0.30 ± 0.02 mg/dl ($P < 0.01$) in group I. Ischemia and reperfusion significantly elevated BUN and serum creatinine 48 h after reperfusion, implying acute renal failure. The i.v. administration of non-immune F(ab′)2 (group III) had no effect on increases of either marker. Treatment with anti-PLA_2-II F(ab′)2 (group IV) significantly reduced the increase of both BUN and serum creatinine compared with the non-immune F(ab′)2-treated group (Fig. 2; BUN values: 26.4 ± 3.5 mg/dl vs. 48.0 ± 8.2 mg/dl ($P < 0.01$); creatinine values: 0.52 ± 0.02 mg/dl vs. 0.73 ± 0.04 mg/dl ($P < 0.01$)). Therefore, it is highly likely that PLA_2-II is involved in injury due to ischemia and reperfusion in kidneys.

Kidneys excised after 48 h were examined histologically by staining with hematoxylin and eosin (data not shown). Non-immune F(ab′)2-treated animals showed severe tubular necrosis, while the tubules remained fairly intact in anti-PLA_2-II F(ab′)2-treated animals. These findings were consistent with results of BUN and serum creatinine level experiments.

3.3. PLA_2 activity in the kidney

PLA_2 activity was assessed to see if the enzyme activity in the kidney was suppressed by administration of anti-PLA_2-II F(ab′)2 (Fig. 3A). PLA_2 activity of the kidney extracts in saline-treated animals with ischemia and reperfusion (group II) was 0.45 ± 0.04 nmol/min/mg vs. 0.29 ± 0.02 nmol/min/mg ($P < 0.01$) in sham-operated animals (group I). PLA_2 activity in kidney extracts significantly increased 48 h after ischemia and reperfusion. The elevation of PLA_2 activity after ischemia was cancelled by pre-incubation with anti-PLA_2-II F(ab′)2 (100 μg/ml) in vitro (Fig. 3B). Thus, the major part of the increase was due to PLA_2-II enzyme. Treatment with non-immune F(ab′)2 (group III) had no effect on kidney PLA_2 activity. Treatment with anti-PLA_2-II F(ab′)2 (group IV) significantly reduced the increase of kidney PLA_2 activity compared with non-immune F(ab′)2-treated animals (Fig. 3A; 0.26 ± 0.02 nmol/min/mg vs. 0.50 ± 0.06 nmol/min/mg ($P < 0.01$)). Therefore, the increase of PLA_2 activity caused

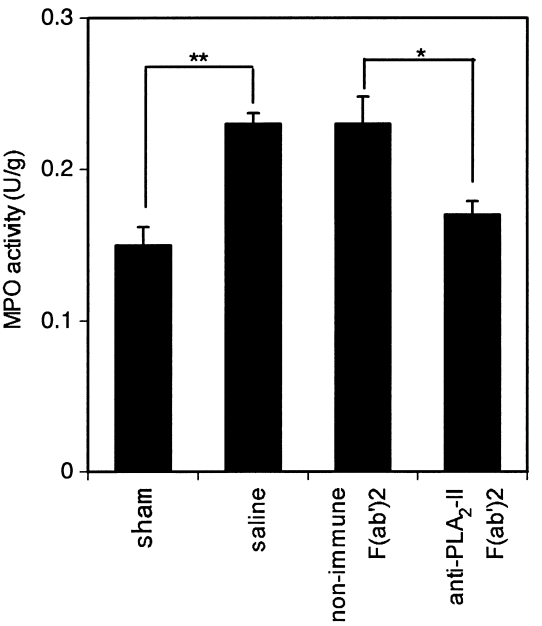

Fig. 4. MPO activity in the kidneys 48 h after ischemia and reperfusion. MPO activity of the kidney extracts is expressed as means ± S.E.M.($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$.

by PLA_2-II enzyme in kidney after ischemia and reperfusion injury was inhibited by administration of anti-PLA_2-II F(ab′)2.

3.4. MPO activity in the kidney

Neutrophil infiltration during ischemia and reperfusion has been implicated as a key mediator in renal tissue injury. But infiltration of neutrophils in kidney is not a prominent feature of human ischemic acute renal failure or experimental acute renal failure when tissue is assessed using routine histologic stains [15,16]. In contrast, assays for neutrophil-derived enzymes, such as MPO, reveal a robust neutrophil existence in ischemic kidney [14,18]. In this experiment, MPO activities in the kidney were measured as a marker of neutrophil infiltration (Fig. 4). The enzyme activities in saline-treated animals with ischemia and reperfusion (group II) was 0.23 ± 0.01 U/g vs. 0.15 ± 0.01 U/g ($P < 0.01$) in sham-operated animals (group I), suggesting that neutrophils infiltrated into kidney tissue damaged by ischemia and reperfusion. Treatment with non-immune F(ab′)2 (group III) had no effect on kidney MPO activity. The increase of kidney MPO activity was significantly reduced in anti-PLA_2-II F(ab′)2-treated animals (group IV) compared with non-immune F(ab′)2-treated animals (Fig. 4; 0.17 ± 0.01 U/g vs. 0.23 ± 0.01 U/g ($P < 0.05$)). These results suggest that neutrophils infiltrate into damaged kidney tissue and that PLA_2-II is involved in the neutrophil infiltration process.

4. Discussion

The present study demonstrated that administration of anti-PLA_2-II F(ab′)2 prevents increases in BUN and serum creatinine, which are indicators of glomerular filtration rate dysfunction caused by tubular necrosis, after ischemia and reperfusion injury.

Types I, II and IV have been identified in rat kidney tissue [2,3]. Anti-PLA_2-II F(ab′)2 is capable of totally inhibiting type II PLA_2 but not type I. Since 100 kDa F(ab′)2 does not penetrate the cell membrane, it is not possible for anti-PLA_2-II F(ab′)2 to inhibit cytosolic PLA_2 (PLA_2-IV) in vivo. The protective effect of anti-PLA_2-II F(ab′)2 on renal injury due to ischemia and reperfusion would result from inhibition of type II PLA_2 but not inhibition of either type I or IV.

To assess the PLA_2 activities in the kidney in this study, the enzyme was extracted from the kidney with acid because type II is resistant to low pH unlike types I and IV [13,18]. PLA_2 activities of acid extracts were significantly elevated 48 h after ischemia and reperfusion and the elevation was inhibited by administration of anti-PLA_2-II F(ab′)2. It is confirmed that the elevation was also inhibited by addition of exogenous anti-PLA_2-II F(ab′)2 in vitro. Thus, the elevated PLA_2 activities appear to be due to an increase of type II enzyme. Nakamura and his co-workers [6] previously showed that renal ischemia and reperfusion resulted in an increase of PLA_2 activity associated with a soluble 14 kDa protein. Also, exposure of rat renal proximal tubules to hypoxia resulted in activation of a Ca^{2+}-dependent 15 kDa PLA_2 and its release out of cells [7]. However, they did not determine whether it was PLA_2-II or other types. The present study has revealed for the first time that renal ischemia and reperfusion injury induces activation of PLA_2-II in the kidney and that inhibition of the enzyme reduces renal tissue damage.

The finding that anti-PLA_2-II F(ab′)2 reduced MPO activity in renal ischemia and reperfusion suggests that the protective effect of anti-PLA_2-II F(ab′)2 results from suppression of neutrophil infiltration. In renal ischemia and reperfusion injury, neutrophils mediate tissue injury through release of oxygen radicals, proteases, arachidonic acid metabolites and other cytotoxic species [5,19]. Neutrophil infiltration in kidney tissue after ischemia and reperfusion is regulated by interaction of the neutrophil CD11a/b with intercellular adhesion molecule 1 (ICAM-1) of endothelial cells [16,17,20]. It was showed that PLA_2-II induces expression of Mac-1 (CD11b/CD18) on human neutrophils through free fatty acid production [10]. PLA_2-II also induces expression of ICAM-1 on endothelial cells through lysophospholipid production [21]. In addition, it is suggested that the increase in free fatty acids and lysophospholipids after ischemia and reperfusion is correlated with the increase in Ca^{2+}-dependent, low molecular PLA_2 activity [6,7]. Thus, neutrophil infiltration may be induced by the degradation products of phospholipids, which is produced by PLA_2-II. Systemic levels of tumor necrosis factor α (TNF-α) and interleukin 1 (IL1), two cytokines known to induce PLA_2-II expression [1,4], is elevated after renal ischemia and reperfusion [19]. TNF-α and IL1 also up-regulate Mac-1 surface expression [22], and PLA_2-II and cytokines have synergistic effect on Mac-1 surface expression [11], thus PLA_2-II induced by cytokines may augment cytokine-induced neutrophil activation by positive feedback. These findings suggest that PLA_2-II induced by ischemia and reperfusion directly and/or indirectly modulates the activation of neutrophils, which infiltrate into the kidney and produce cytotoxic substances leading to tissue injury.

In contrast to the findings described above, PLA_2-I had a cytoprotective effect on isolated rat proximal tubules which were subjected to hypoxia and reoxygenation, even though the concentrations of free fatty acids were increased by the addition of PLA_2-I [23]. There has been no evidence of such a cytoprotective effect with PLA_2-II. Our finding in renal ischemia and reperfusion with PLA_2-II-specific antibodies supports the thesis that type II enzyme works in vivo as a pathogenic factor rather than a protective factor.

Since anti-PLA_2-II F(ab′)2 ameliorated acute renal failure caused by ischemia and reperfusion, antibodies against PLA_2-II could be a useful therapeutic tool to acute renal failure caused by ischemia.

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